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Adenylyl Cyclase Activities in Ovarian Tissues. II. Regulation of Responsiveness to LH, FSH, and PGE_1 in the Rabbit

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ABSTRACT. We conducted a study to determine whether a decline in the capacity of preovulatory follicles to accumulate cAMP in response to *in vitro* LH exposure, reported by Marsh *et al.* (1), is due to direct desensitization of the LH-hCG-responsive adenylyl cyclase (AC) system. Enzyme activity was determined in the absence and in the presence of 10 μg/ml of LH, FSH, and prostaglandin (PG) E_1 in homogenates prepared from Graafian follicles dissected before and at various times after rendering them preovulatory either by mating (endogenous LH release) or by injection of hCG (100 IU/3.5–4.5 g rabbit, iv). Both of these treatments resulted in desensitization of the AC to LH and FSH stimulation, so that by the time of ovulation these responses were absent. hCG-induced desensitization was rapid (50% loss of LH-stimulated activity was obtained within 5 min of injection), was dose-dependent, requiring an ovulatory dose; was selective for LH- and FSH-stimulated activity, being without effect on basal and PGE_1 as well as NaF-stimulated activities; and was induced specifically by LH (mating) and hCG—FSH and PRL being without effect. Desensitization of follicular AC does not seem to be mediated by PG, for doses of indomethacin that inhibited ovulation did not interfere with the loss of LH responsiveness. The incubation *in vitro* of dissected Graafian (estrous) follicles with LH (2 h) also resulted in desensitization to LH stimulation and indicated that this phenomenon was due to a direct effect of LH on the follicles and was not mediated by hormones derived from other endocrine structures.

Newly formed corpora lutea (CL) in either pregnancy or pseudopregnancy (PSP) developed a new AC system that was highly responsive to LH and less responsive to FSH and PGE_1. This new system persisted for the duration of pregnancy and PSP, and became inactive with regression of CL. The prolongation of PSP by hysterectomy was accompanied by the persistence of an active LH-responsive AC system. The injection of ovulatory doses of hCG into 6-day PSP rabbits produced, within 2 h, a 50% desensitization of the luteal AC systems to LH stimulation.

After day 10 of pregnancy, the levels of LH-stimulated AC activity in CL and the levels of progesterone in serum, reported by Challis *et al.* (2), were found to parallel each other, suggesting that this enzyme activity may be a physiologic regulatory step in progesterone synthesis by the normal CL throughout most of the animal's pregnancy. This finding is discussed in the light of compelling evidence that estrogens are luteotrophic in the rabbit. (*Endocrinology* **99**: 185, 1976)

AN OVULATORY dose of LH or human (h)CG presented to rabbit Graafian follicles induces morphological as well as biochemical changes, culminating in 10 to 11 h in ovulation and the formation of a corpus luteum (CL). During this preovulatory period, the following changes appear to be initiated by LH: steroids (especially estrogens, testosterone, and 20α-hydroxypregn-4-en-3-one) are poured into the follicular fluid and the ovarian vein for approximately 4 h (3–7); RNA and protein synthesis increase (8,9); peptidase activity increases (10); follicular pressure changes occur (10); prostaglandin (PG) synthesis increases in the last 4 h before ovulation (11–13); meiotic division in the oocyte is induced (14); and such morphological changes as the decomposition of connective tissue surrounding the follicle and a thinning in the outer follicular wall occur (10). Work has been carried out in recent years on the mechanism by which LH exerts its multiple actions on the ovary, and, although

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it is not fully proven, many of the mechanisms seem to be mediated by cyclic AMP (cAMP). This nucleotide mimics the steroidogenic effects of LH in luteal and interstitial tissue (15–17), the luteinizing effects of LH in incubated granulosa cells (18,19), and the initiation of the first meiotic division of oocytes (14). In 1973, 2 laboratories working with Graafian follicles reported that while LH or hCG initially stimulated cAMP accumulation, these follicles soon afterwards exhibited a decreased ability to accumulate cAMP. Marsh *et al.* (1) reported that Graafian follicles from estrous rabbits rapidly lost their capacity to accumulate labeled cAMP from [8-^{3}H]-adenine between the time of hCG injection (iv) and ovulation. Similarly, Lamprecht *et al.* (20) showed that proestrous rat follicles incubated 18 h with LH lost their capacity to increase cAMP formed from prelabeled ATP in response to an acute (20 min) presentation of LH, without losing their capacity to increase cAMP in response to PGE_2.

It was the initial purpose of this work to explore, using direct adenylyl cyclase determination, the possibility that the decrease in cAMP-accumulating capacity after hCG treatment, reported by Marsh *et al.* (1), was a direct effect on the adenylyl cyclase (AC) system.

As will be shown below, we found desensitization to be a direct effect on AC not only in estrous follicles, as predicted by the studies of Marsh *et al.* (1) and Lamprecht *et al.* (20), but also in CL. We report characteristics of this desensitization process as seen *in vivo* and *in vitro* (hormonal specificity, selectivity, and dose-dependency) and present data on the development of gonadotrophin- and PG-stimulated adenylyl cyclase in the CL of pregnant and pseudopregnant rabbits.

Materials and Methods

Animals

Rabbits (3.5–4.5 kg), primarily New Zealand Whites, which had littered at least one time, were housed in air-conditioned quarters and allowed free access to water and a commercially pelleted food. Females which were neither pregnant nor pseudopregnant were considered to be in estrus. Pseudopregnancy (PSP) was induced with the injection of 100 IU hCG (Ayerst) dissolved in 0.5 ml of 0.9% saline, into a peripheral ear vein. Pregnancy was induced by successive mating to two different bucks in order to insure ovulation. The day following mating or PSP induction was counted as day 1.

Hysterectomy was performed either 10 days prior to or 7 days after the induction of PSP as described by Spies *et al.* (21).

The rabbits were sacrificed by cervical dislocation between 1200 and 1500 h unless otherwise specified. The ovaries were removed and immediately cooled to 0 C in iced Krebs-Ringer Bicarbonate buffer (KRB). The follicles were dissected using Graefe forceps (Roboz Surgical Co.), rendered free of interstitial tissue, and kept in iced KRB until homogenization. Microscopic examination of paraffin-mounted follicles (unpopped, fixed in Bouin's solution, and sectioned at 6 μm) revealed that the follicles were free of interstitial tissue and retained variable amounts of theca cells. For the adenylyl cyclase assay, the follicles were popped to remove follicular fluid prior to homogenization and the CL were dissected free of interstitial tissue and kept similarly in iced KRB until homogenization.

Materials

hCG (Ayerst), LH (NIH-LH-B8 and B9; FSH contamination: less than 0.05 U/mg, ref. 22), FSH (NIH-FSH-P1; LH contamination: 0.0075 U/mg, ref. 22), and PRL (NIH-P-S11) were gifts of the Endocrine Study Section, National Institutes of Health, Bethesda, Maryland. Peptide hormones for injection, were dissolved in 0.9% saline. When used in the adenylyl cyclase assay, 1 mg/ml stock solutions of LH, FSH (prepared in 100 mM NaCl), and PGE_1 (from Dr. John Pike, the Upjohn Co., Kalamazoo, Michigan; dissolved as recommended) were diluted freshly to 50 μg/ml with 0.1% bovine serum albumin solution (Fraction V, Armour). Indomethacin was purchased from Sigma Chemical Company and was injected (SC) as a suspension in peanut oil (70 mg/ml). Creatine phosphate and creatine kinase were obtained from Calbiochem. [α-^{32}P]-ATP was obtained from New England Nuclear and ICN Pharmaceuticals, Inc., and was used

without further purification. Tris-ATP and cAMP were obtained from Sigma Chemical Company.

In vitro *incubation of follicles*

Ovaries were removed from estrous rabbits and placed in 0.9% saline at room temperature. Unpopped follicles measuring about 2 mm in diameter were then dissected and returned to the saline until 25–30 follicles had been dissected (usually 4 rabbits). Five to six follicles were placed in each of 5 vials containing 2.5 ml KRB and 1 mg/ml glucose at 37 C, or in KRB-glucose containing either bovine serum albumin (BSA, Fraction V, charcoal purified) or LH (each diluted from 1 mg/ml stock with KRB-glucose). The vials were closed with stoppers having two 20-gauge needles and were incubated for 2 h in a metabolic shaker at 37 C, with each vial continuously receiving, by means of tubing connected to one of the needles, a warmed and humidified gas mixture of 95% O_2 and 5% CO_2. After 2 h, the follicles were first blotted dry and then washed by placing them in KRB-glucose (containing no addition) at 37 C and incubating them for 15 min. The incubation was terminated by placing the vials on ice, popping the follicles, and homogenizing them for the adenylyl cyclase assay.

Adenylyl cyclase assay

The dissected follicles or CL, which had been kept in iced KRB until the dissections were completed, were homogenized in 10 volumes (follicles) and 20 volumes (CL) of ice-cold medium containing 27% (wt/wt) sucrose, 1.0 mM EDTA, and 10 mM Tris-HCl, pH 7.5, using 10 strokes with the loose (CL) and 10 strokes with the tight (follicles and CL) pestle of an all-glass Dounce homogenizer (Kontes Cat. no. K-885300, size 7). The homogenate was filtered through no. 12 silk screen, and 20 μl aliquots were assayed for basal and hormonally responsive AC activities.

Adenylyl cyclase activity was determined in a medium containing 3.0 mM [α-^{32}P]ATP, 5.0 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM [^{3}H]cAMP (approximately 10,000 cpm), 25 mM Tris-HCl, pH 7.0, an ATP-regenerating system consisting of 20 mM creatine phosphate and 0.2 mg/ml of creatine kinase at pH 7.0, homogenate protein (at concentrations in the linear range of AC activity), and, when present, 10 μg/ml of LH, FSH, or PGE_1. The final volume of the reaction was 50 μl. The incubations were at 37 C for 10 min and were terminated by the addition of 0.1 ml 10 mM cAMP, 40 mM ATP and 1.0% sodium dodecyl sulfate, followed by boiling for 3.5 min. cAMP was isolated according to the method of Salomon *et al.* (23) using Dowex chromatography (24) and alumina chromatography (25,26) with minor modifications as described elsewhere (27). The radioactivity present in the final eluate, containing [^{32}P]cAMP and [^{3}H]cAMP (added as a marker for recovery) was determined in a Packard Tri-Carb liquid scintillation counter. All tissue samples were incubated in duplicate. The means of such duplicate determinations (usually within 5%) are presented as single points on graphs. When more than one tissue sample was obtained and assayed, the means ± SEM of a number of such assays are presented on graphs. Each tissue sample was derived from the pooled follicles or CL of at least 2 rabbits that had received the same treatment(s).

The results were expressed either as absolute activities in picomoles of cAMP formed per minute per mg of homogenate protein or as stimulated activities relative to basal.

Protein was determined by the procedure of Lowry *et al.* (28) using crystalline BSA as standard.

Results

1. *Experiments with Follicles*

Desensitization of the follicular adenylyl cyclase. The growth of antral follicles in rabbit ovaries was found to be associated with both a decrease of basal AC activity and the development of a marked response to LH and FSH, but not to PGE_1. Thus, in an experiment that compared AC activities in homogenates from estrous rabbit follicles that were smaller than 1.5 mm in diameter, to activities derived from follicles whose size exceeded 1.5 mm, we found that with increase in size, basal activity decreased from 20 ± 0.8 pmoles/min/mg homogenate protein (mean ± SD of an assay in which the follicles from 4 rabbits were used) to 8.3 ± 3.8 (mean ± SEM of 5 assays in each of which the follicles from 3 rabbits were used), while LH- and FSH-stimulated activities increased from 35 ± 1.2 and 31 ± 1.0, re-

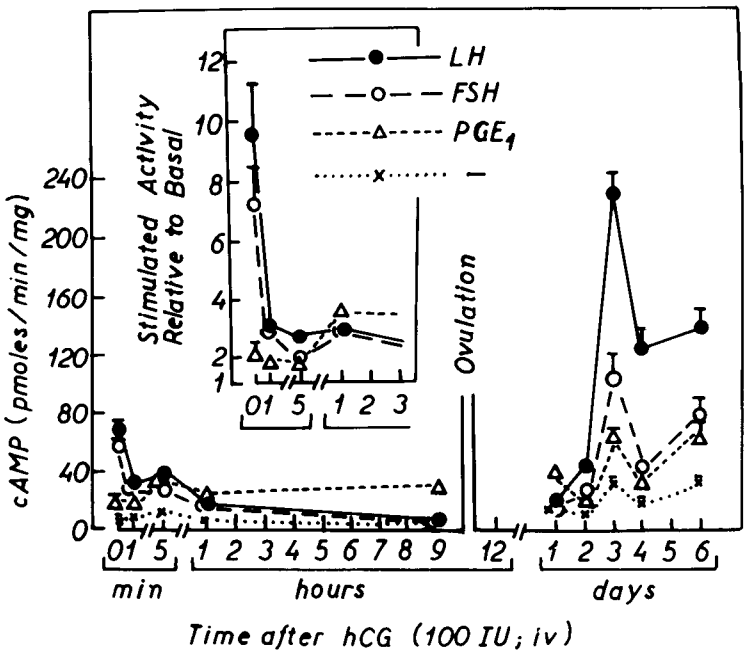

FIG. 1. Adenylyl cyclase activities in homogenates of dissected rabbit ovarian follicles and CL obtained before (time 0, estrous follicles) and after (1 min to 6 days) the injection of hCG (100 IU/3.5–4.5 kg rabbit, iv). The activity was determined in the absence (basal) and presence of 10 μg/ml of LH, FSH, and PGE_1. Both absolute activities (time 0 to 6 days) and relative activities (inset, time 0 to 3 h) are shown. The 1-min point was obtained by injecting the rabbit and immediately killing it by cervical dislocation, removing the ovaries, and placing them in ice-cold KRB. Single points represent one assay in which a minimum of 2 rabbits were used. Mean ± SEM is shown where 2 to 6 such assays were performed. For time 0, 3, 4, and 6 days *n* equals 5, 3, 2, and 6, respectively; for all other points, *n* equals 1. NaF-stimulated activities (in pmoles/min/mg protein) were respectively 70 ± 2 (n = 4), 60, 73, 102, 67, 67, 62, 129 ± 10 (n = 2), 72, 124, and 168 ± 20 (n = 2) at 0, 1, and 5 min, 1, 9, and 24 h, and 2, 3, 4, 5, and 6 days after hCG; values are mean ± SEM when more than one assay was carried out, with the number of such assays given in parentheses; NaF in the incubations was 10 mM.

spectively (relative stimulations of 1.75 and 1.55 times), to 70 ± 11.9 and 58 ± 7.0 (relative stimulations 8.46 and 7.02). The PGE_1 stimulated activity diminished slightly from 33 ± 0.8 (relative stimulation 1.65 times) to 20.0 ± 8.0 (relative stimulation, 2.41). It should be noted that this finding, *i.e.*, a decrease in basal activity with the appearance of increased hormone-stimulated activity, does not appear to be restricted to rabbit Graafian follicles, since it was also observed to occur between days 8 and 11 in the ovaries of immature rats and between noon of diestrus and noon of proestrus in the follicles of cycling rats (29).

Ovulatory doses of hCG (100 IU, iv) result in desensitization of follicular AC to gonadotrophin stimulation, as predicted from the results of Marsh *et al.* and Lamprecht *et al.* (Fig. 1). Within 1 min after the injection of hCG, the gonadotrophin-stimulated activity and the relative responsiveness of the AC decline approximately 70%. The effect of hCG on the cyclase system is selective since the responsiveness of the cyclase to PGE_1 and NaF is not changed. The decline in the responsiveness of AC to LH and FSH is not accompanied by an increased basal activity. As ovulation approaches, the AC becomes less responsive to LH and FSH, so that at the time of ovulation the cyclase is no longer stimulated by LH or FSH, but remains fully responsive to NaF (*ca.* 9-fold stimulation)[3] and to PGE_1 (*ca.* 4-fold stimulation). The cyclase remains desensitized to LH and FSH until 48 h after hCG injection. By 72 h, the cyclase in the newly formed CL is no longer desensitized to gonadotrophins and, in fact, exhibits an LH-stimulated AC activity which is 4-fold greater than that in estrous follicles, while the FSH-, PGE_1-

[3] While this article was being reviewed, Nugent *et al.* (57) confirmed our findings that gonadotrophin-induced desensitization of ovarian adenylyl cyclase of gonadotrophin stimulation occurs without loss of NaF-stimulated activity.

and NaF-stimulated activities of this luteal cyclase equal those in the follicle.

When ovulation is induced by mating *via* an endogenous LH surge, the LH- and FSH-stimulated AC activities decline rapidly, following a pattern of desensitization that is equivalent to that seen following hCG injection (Fig. 2). The PGE_1- and NaF-stimulated AC activities are unchanged under these conditions, indicating that the enzyme is rendered selectively unresponsive.

Dose dependence of desensitization. This experiment was designed to determine whether the follicular AC system could be desensitized by doses smaller than ovulatory ones. In a pilot experiment, we found that for hCG to be ovulatory the dose had to exceed 5 IU/kg body weight. The relationship between the dose of hCG injected and the loss of responsiveness of the cyclase to LH after 2 h (Fig. 3) shows that desensitization requires doses that are ovulatory.

In vitro *desensitization of the adenylyl cyclase system.* This experiment was designed to determine whether it is possible to obtain desensitization of the AC system to gonadotrophins *in vitro* as efficiently as *in vivo*. To this effect, unpopped rabbit follicles were incubated for 2 h in KRB-glucose in the presence of BSA or in 3 different concentrations of LH. The incubation of the follicles with BSA resulted in a sharp and, as yet, unexplained decrease of both basal and PGE_1-stimulated AC activities. However, the activities determined in the presence of LH or FSH remained unchanged, indicating that both catalytic capacity and hormonal responsiveness to gonadotrophin were not adversely affected. Incubation with 0.8 μg/ml LH mimicked the desensitization of follicular AC obtained *in vivo*. While 8 μg/ml LH also decreased the LH- and FSH-stimulated activities of the cyclase, basal activity increased, suggesting receptor occupation at this LH dosage. Incubation with 0.1 μg/ml LH produced no desensitization, showing that a dose dependency is maintained *in vitro* (Fig. 4).

Specificity of follicular desensitization. Estrous rabbits were injected with oPRL (100 μg/3.4–4.5 kg rabbit) or pFSH (250 μg/3.5–4.5 kg rabbit); ovulation was checked after 24 h in some, and the responsiveness of the cyclase to LH, FSH,

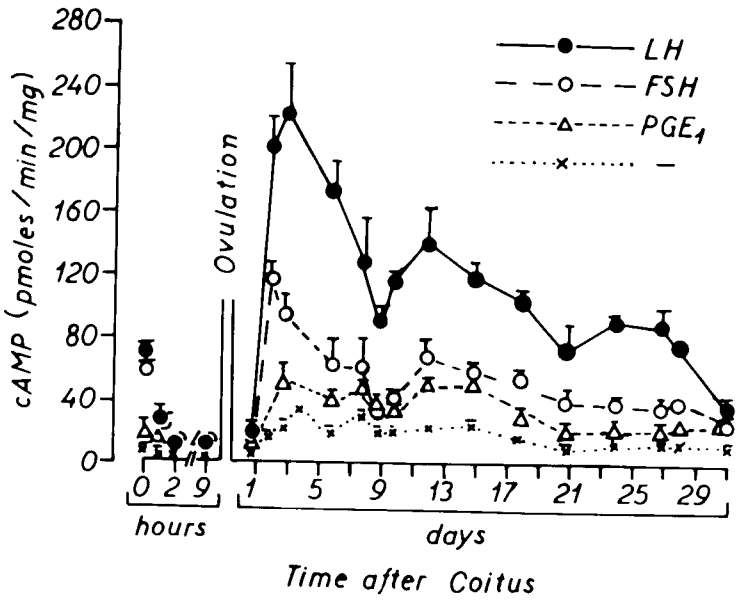

FIG. 2. Adenylyl cyclase activities in homogenates of dissected rabbit ovarian follicles and CL obtained before (time 0, estrous follicles) and after (1 h to 31 days) mating each female to 2 fertile bucks in succession. For the rest of the conditions see legend to Fig. 1. For time 0, 2 h, 1, 2, 3, 4, 6, 8, 9, 10, 12, 15, 18, 21, 24, 27, and 31 days, *n* equals 5, 2, 2, 2, 3, 2, 2, 2, 2, 2, 3, 2, 2, 2, 2, 2, and 2, respectively; for all other points, *n* equals 1.

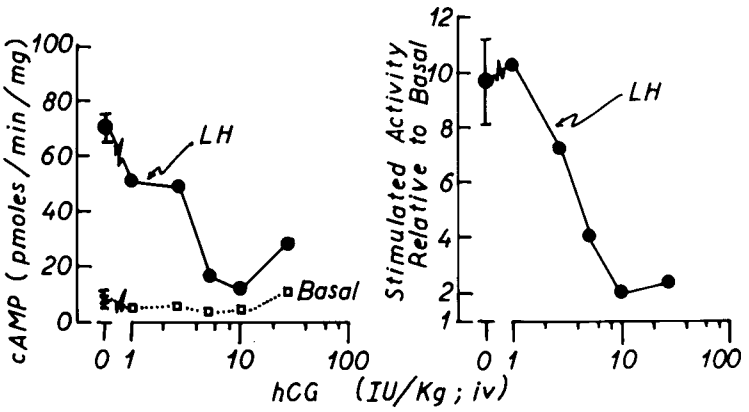

FIG. 3. Effect of the dose of hCG on the responsiveness of adenylyl cyclase to LH. Both absolute and relative activities are shown. For determination of activities, rabbit follicles were dissected and homogenized 2 h after injection of the indicated doses of hCG. "O" hCG represents follicles from estrous rabbits which received no hCG injection. For time 0, n equals 5; for all other points, n equals 1.

and PGE_1 was tested in others after 2 h. We found that while neither PRL nor FSH induces ovulation, both produced some decline in LH-, FSH-, and PGE_1-stimulated AC activities. However, basal activities were depressed to the same degree, resulting in *no* significant desensitization by PRL or FSH if activities were expressed relative to basal. The probable interactions of PRL and FSH with the AC system are not clear and require further investigation.

Role of PG in desensitization of the follicular adenylyl cyclase system. Since indirect evidence has linked cAMP synthesis to PG and since PG increases dramatically in the last few hours prior to ovulation (10–12) and may increase locally even earlier, we sought to determine whether PG had a role in the loss of responsiveness of the follicular AC to LH and FSH, by testing whether indomethacin, a blocker of PG synthesis, would interfere with the desensitizing effects of hCG. The pretreatment of estrous rabbits with indomethacin (70 mg, SC) 2 h before an hCG injection, did not alter the desensitization of the cyclase to LH and FSH observed 2 h after hCG administration (Fig. 5). It is interesting that indomethacin appeared to decrease PG-stimulated activity, but this effect was not investigated further. Since ovulation is not disturbed with this indomethacin treatment (30), but is blocked when indomethacin is administered twice, when hCG is given and again 8 h after hCG, the desensitizing effect of hCG was also tested with this second regimen of indomethacin treatment. This treatment was also found to be ineffective in interfering with hCG action on AC, indicating that hCG-induced loss of responsiveness of the follicular AC to LH and FSH stimulation is not mediated by PG. As indicated (legend to Fig. 5), control injections of indomethacin followed by saline, rather than by hCG, had no significant effect on the LH-sensitive AC activity of estrous follicles.

2. *Experiments with Corpora Lutea*

Patterns of adenylyl cyclase activities throughout PSP and pregnancy. In the CL of pregnancy, the LH-stimulated activity of the AC system seems to develop in 3 stages (Fig. 2), characterized by 3 maxima on days 3, 12, and 25, respectively. Two minima occur, one on day 9 (2 days after implantation) and the other on day 21.

The CL of PSP (Fig. 6) undergo a developmental pattern, similar to that of pregnancy, with a maximum on day 3; however, the LH-stimulated AC activity increases 24 h earlier in pregnancy than in hCG-induced PSP, as seen on day 2. Until the first minimum on day 10, a more constant level of LH-stimulated AC is maintained in PSP than is found in pregnancy. The CL of PSP also exhibit the second stage of LH-stimulated AC activity that is found in pregnancy.

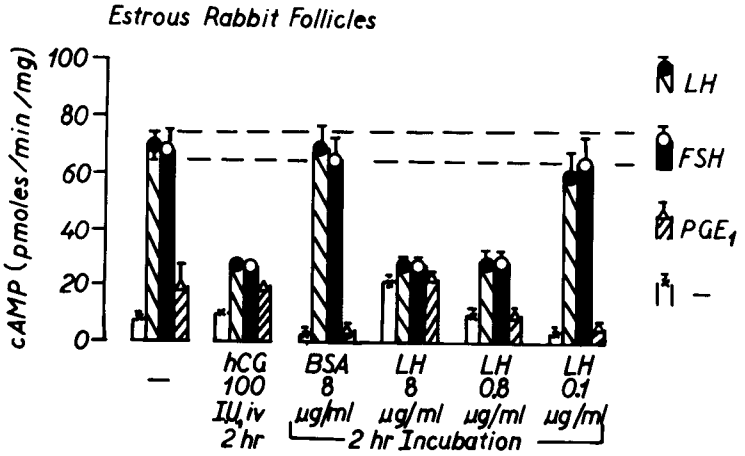

FIG. 4. Effect of *in vitro* exposure of rabbit follicles to BSA or LH on the responsiveness of the adenylyl cyclase. Dissected follicles were incubated at 37 C for 2 h with continuous gassing (95% O_2/5% CO_2) in the presence of BSA (8 µg/ml) or LH (8, 0.8, or 0.1 µg/ml). The follicles were then homogenized and adenylyl cyclase activity was determined in the absence and presence of 10 µg/ml LH, FSH or PGE_1. "–" represents follicles from estrous rabbits which were not incubated. For estrous follicles (not incubated), *n* equals 5; for BSA, 8 µg/ml LH, 0.8 µg/ml LH, and 0.1 µg/ml LH, *n* equals 5, 3, 2, and 2, respectively.

The CL of PSP and pregnancy exhibited basal AC activities which were *ca.* 3-fold higher than those of estrous follicles (Figs. 2 and 6).

Desensitization of the adenylyl cyclase system in rabbit CL and specificity for hCG. Experiments by Stormshak and Casida (31) and Spies *et al.* (21) have shown that the injection of ovulatory doses of hCG into PSP rabbits results in luteolysis. We sought to determine whether the luteolytic effect of hCG was reflected in the luteal AC. An ovulatory dose of hCG injected 2 h prior to the sacrifice of a 6-day PSP rabbit resulted in the desensitization of the luteal AC to LH with no effect on the FSH or PGE_1 responses (Fig. 7) or on the response to NaF (see legend to Fig. 7). Desensitization of the system to LH was induced only with hCG; FSH and PRL were without effect. However, FSH, but not hCG or PRL, seems

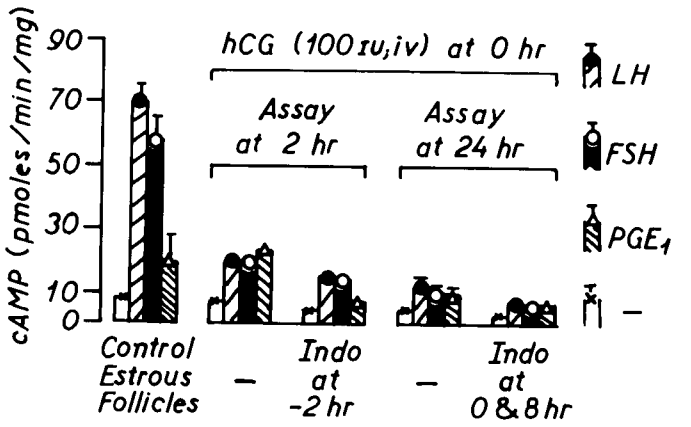

FIG. 5. Lack of effect of indomethacin on the loss of responsiveness to LH of adenylyl cyclase in rabbit follicles obtained 2 or 24 h after the injection of hCG. Indomethacin (70 mg/ml/3.5–4.5 kg rabbit, SC) was injected either 2 h before hCG (100 IU/3.5–4.5 kg rabbit, iv) or within and 8 h after hCG. Adenylyl cyclase activities in homogenates of follicles were determined in the absence and presence of 10 µg/ml LH, FSH, or PGE_1. For control estrous follicles, *n* equals 5; for CL obtained 24 h after hCG injection (–), *n* equals 2. The substitution of saline for hCG resulted in the following activities: 5.1 ± 0.2 (basal) and 66.8 ± 2.8 (LH-stimulated) when tested in follicles from rabbits that had received indomethacin at −4 h and saline at −2 h (mean ± SEM in pmoles/min/mg protein of 2 assays, each of which used follicles from 3 rabbits), and 7.1 ± 1.1 (basal) and 60.4 ± 2.3 (LH-stimulated) when tested in follicles from rabbits that had received indomethacin and saline at −24 h and indomethacin at −16 h (mean ± SD in pmoles/min/mg protein of 1 assay in which follicles from 3 rabbits were used).

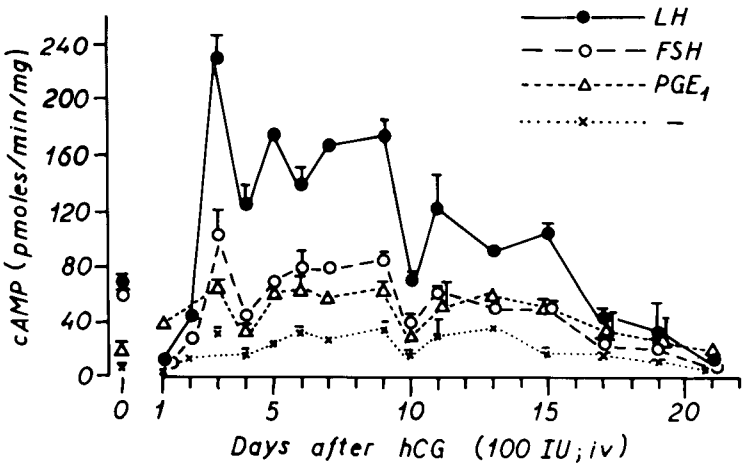

FIG. 6. Adenylyl cyclase activities in homogenates of dissected rabbit ovarian follicles and CL obtained before (time 0, estrous follicles) and after (1 day to 21 days) injection of hCG (100 IU/3.5–4.5 kg rabbit, iv). Single points represent one assay in which a minimum of 2 rabbits was used. Mean ± SEM is shown where 2 to 6 such assays were performed. For days 0, 1, 3, 4, 6, 9, 10, 11, 15, 17, 19, and 21, n equals 5, 2, 3, 2, 6, 2, 4, 2, 3, 2, 2, and 2, respectively; otherwise, n equals 1.

to increase PG-stimulated activity, an effect that will require further investigation.

Effect of hysterectomy on the CL adenylyl cyclase system. Since hysterectomy extends the functional life of the CL of PSP rabbits (21,32,33), we checked to see whether the AC activity was also maintained. Figure 8 shows that hysterectomy, whether it is performed before or after the induction of PSP, results in LH-stimulated AC activities that are notably higher than those from the CL of control PSP rabbits (see Fig. 6), indicating that the maintenance of PSP by hysterectomy is accompanied by the maintenance of a responsive AC system.

In other experiments we checked to see whether the homogenate obtained from a desensitized CL contained a factor which had been liberated into the medium and which was responsible for the desensitizing effect. The incubation of an equimixture of homogenates from desensitized CL and control CL resulted in no further decline in the sensitivity of the cyclase to LH other than that attributable to the desensitized CL (Table 1), indicating that the loss of responsiveness of the luteal cyclase is not

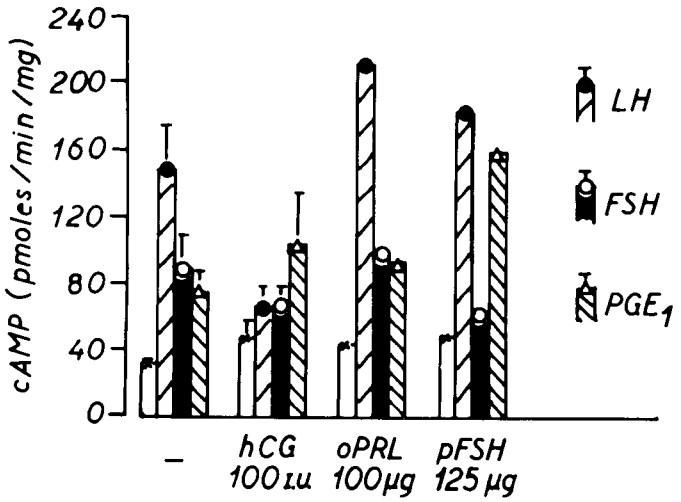

FIG. 7. Effects of hCG, PRL, or FSH on the responsiveness of adenylyl cyclase to LH, FSH, and PGE_1 in CL from 6-day PSP rabbits. CL were dissected and homogenized 2 h after injection of hCG (100 IU/3.5–4.5 kg rabbit; iv), PRL (100 µg/3.5–4.5 kg rabbit, iv), or FSH (125 µg/3.5–4.5 kg rabbit, iv). For 6-day-old CL before and after hCG injection, n equals 6. NaF-stimulated activity, determined in two instances in homogenates of hCG-treated animals (6 day PSP), was 171 ± 7 pmoles/min/mg protein, not significantly different from activity found before hCG injection (see legend to Fig. 1).

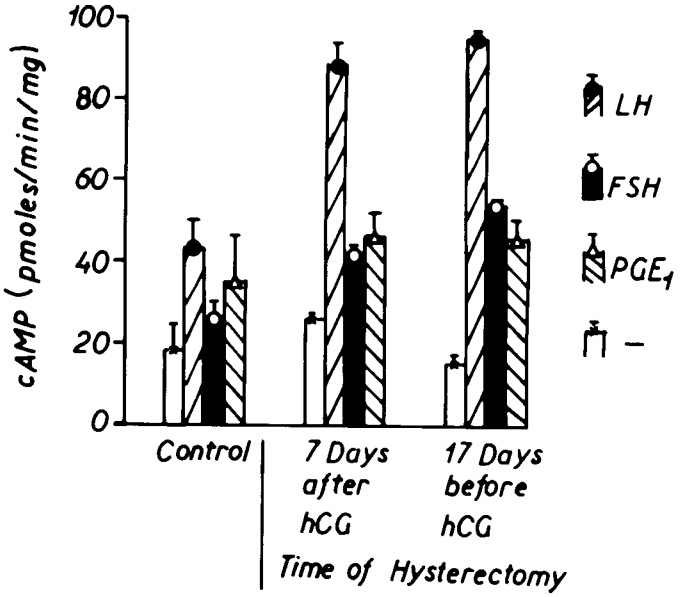

FIG. 8. Effect of hysterectomy on the responsiveness of adenylyl cyclase in homogenates of CL obtained from 17-day PSP rabbits. Hysterectomies were performed 7 days after or 17 days before the induction of PSP with hCG (100 IU/3.5–4.5 kg rabbit, iv). For control CL (same data as shown on Fig. 6) and for CL obtained from rabbits which were hysterectomized 7 days after and 17 days before hCG injection (initiation of PSP), n equals 2, 2, and 2, respectively.

due to the accumulation of an inhibitor of cyclase activity.

Discussion

Stimulatory hormones often promote only a transient elevation of cAMP levels. One way in which the cAMP levels can be lowered is by the desensitization of the adenylyl cyclase system to its own stimulant. Two laboratories reported in 1973 that Graafian follicles exposed to LH or hCG lose their capacity to accumulate cAMP, presumably by a direct effect of LH and hCG on the follicular AC system (1,20). We have confirmed this assumption by showing that LH and hCG directly induce the desensitization of AC to LH in Graafian follicles from estrous rabbits and in CL from PSP rabbits without major increases in basal activities. The desensitization of AC as a means of reducing cAMP levels does not appear to be restricted to ovarian structures. Human fibroblasts (34) lost their adenylyl cyclase response to catecholamines and PG when pre-incubated with catecholamines or PG, respectively, with little or no cross-over effect. A similar finding was reported for guinea pig macrophages (35), and it seems reasonable to assume that the desensitization of AC, like the stimulation of AC, is a receptor-mediated phenomenon.

The mechanism by which the AC is desensitized is not understood, but the

TABLE 1. Absence of inhibitory factor in homogenates of desensitized corpora lutea

Volume of homogenate from		Adenylyl cyclase activity due to LH addition*	
Control CL	Desensitized CL	Found†	Calculated
(μl)		(pmoles/10 min)	
10	—	73.2 ± 5.8	—
20	—	164.7 ± 6.2	—
—	10	32.3 ± 2.1	—
—	20	64.5 ± 5.3	—
10	10	110.6 ± 9.9	105.4 ± 6.2

* Homogenates were pre-incubated for 10 min at 30 C before their adenylyl cyclase activity was determined.

† Values are mean ± standard deviation of the difference in activities obtained between triplicate incubations for basal and duplicate incubations for LH-stimulated adenylyl cyclase activities. Basal activities (mean ± SD) for the 6-day-old control and desensitized CL used in this experiment were 35.6 ± 2.4 and 59.2 ± 1.4 pmoles per min per mg, respectively. Ten μl of the homogenate of control and desensitized CL contained 99 and 86 μg protein, respectively.

finding of De Vellis and Brooker (36) that development of refractoriness to catecholamines in rat glioma cells is inhibited by RNA and protein synthesis inhibitors suggests the involvement of a rapidly turning-over protein. As reported elsewhere (27) studies with isolated membranes from pig Graafian follicles, showing that the desensitization of AC to LH stimulation is a process that depends strictly on the addition of ATP and magnesium ions, suggest that phosphorylation of one of the cyclase components may be the biochemical basis for the change leading to desensitization.

From a physiologic point of view, changes of responsiveness appear to correlate with steroidogenic activity in both follicles and CL of the rabbit. Thus, steroid levels rise following coitus or hCG injection to a maximum at about 4 h after treatment and then decline to virtually unmeasurable concentrations prior to ovulation (3,4,6,38). Steroidogenesis is not resumed until some 48 h after the induction of ovulation, at which time progestin levels gradually increase. The addition again of hCG around the time of ovulation elicits *no* steroidogenesis (37). While this cessation of steroidogenesis prior to, during, and after ovulation has been related to decreased steroid precursor availability (38), Mills *et al.* (37) have suggested that a more serious impairment of the metabolic capabilities of the differentiating follicle (CL) is involved. However, since during this period of quiescent steroidogensis the response of adenylyl cyclase to LH is practically absent (Figs. 1,2 and 6), it is possible that this may be the actual reason for reduced steroid synthesis. Thus, desensitization would halt steroidogenesis by preventing gonadotrophin action until the AC system becomes again responsive to gonadotrophins.

It is not clear which cell type (granulosa and/or theca cells) associated with the Graafian follicle contains the AC system which is desensitized to LH. Although both theca cells (39,40, Hunzicker-Dunn and Nalbandov, personal observation) and granulosa cells (17,41) possess specific binding sites for LH and hCG, and presumably contain adenylyl cyclases stimulated by LH, the resolution of which cell type contains the AC system that is desensitized by LH or hCG, awaits a technique for successfully separating these cell types.

We superimposed progesterone levels in pregnant rabbits, as reported by Challis

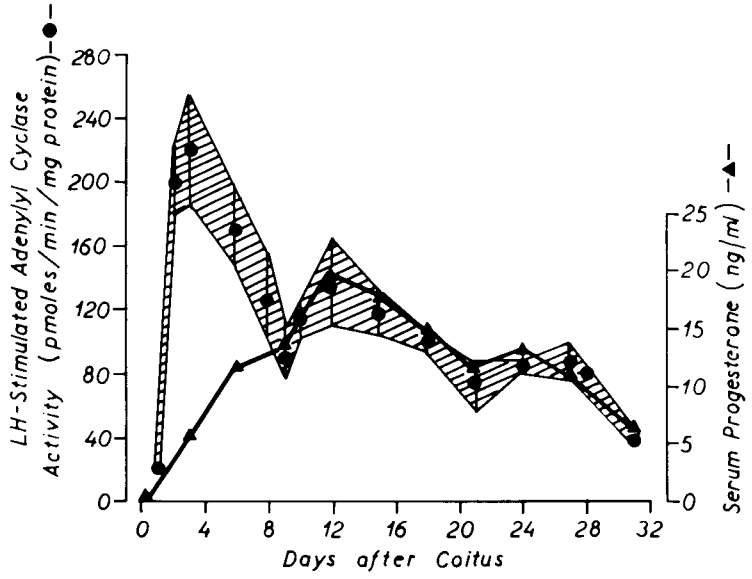

FIG. 9. Relationship between LH-stimulated adenylyl cyclase activities in homogenates of dissected ovarian CL, and serum progesterone levels (Challis *et al.*) (2), obtained 1 to 31 days after mating. For details of adenylyl cyclase activity, see legend to Fig. 2.

et al. (2), upon our LH-sensitive AC data and obtained Fig. 9. This comparison showed that the LH-stimulated adenylyl cyclase appears to correlate with progesterone levels after about 10 days of pregnancy, but not before this time. The striking correlation between LH-stimulated cyclase activity and progesterone levels after day 10 (Fig. 9) suggests that LH may play a major role in the control of steroid production by the rabbit CL. This suggestion would shift the emphasis away from estrogen, which has been assigned the role of the primary luteotrophic agent in the rabbit. The importance of estrogen has been based on three lines of evidence. a) The removal of follicles during pregnancy induces morphological luteolysis, decreases progesterone secretion, and causes abortion, and estrogen replacement therapy prevents all of these deleterious effects (42–46). b) Pregnancy can be maintained (50%) in hypophysectomized rabbits with estrogen treatment (47). c) Rabbit CL possess estrogen receptors (48,49). Furthermore, while an absolute requirement for LH in luteal function has been accepted based on the finding that LH antiserum induced abortion after 3 days of pregnancy (50), this luteotrophic function of LH has been believed to be an indirect one, operating through the stimulation of follicular estrogen secretion. On the other hand, evidence showing that LH stimulates AC activity in the dissected CL (51), and that hCG-induced desensitization of CL AC (Fig. 6) can be obtained also after cauterization of the antral follicles[4] and before ovarian levels of estrogen would be expected to diminish below control levels, strongly argues for an *in vivo* LH effect directly on the CL. This, coupled to the striking correlation between LH-stimulated AC activity and progesterone levels during pregnancy (after implantation) suggests that it is possible that the luteal AC is a regulatory (*i.e.*, rate-limiting) step in steroidogenesis under "normal" conditions (*i.e.*, in the estrogen-supported CL), with LH regulating cAMP formation and estrogen being responsible for the maintenance (induction) of adequate levels of both LH-dependent AC and steroidogenic enzymes. Steroid synthesis enzymes would all be present due to "trophic" estrogen levels in the normal pregnant rabbit (hence the estrogen requirement), but would be active only to a low degree, producing insufficient levels of progesterone to maintain pregnancy adequately in the absence of LH. Thus, we hypothesize that a luteotrophic complex consisting of the combination of estrogen *and* LH is probably necessary to maintain *normal* luteal function in the rabbit. In fact, as recently demonstrated by Holt *et al.* (53), normal PSP in the rabbit is not associated with limiting estrogen supply to the CL, for 2 to 3-fold chronic elevations of serum estrogen levels, for as long as 12 days by implantation of estradiol-containing Silastic capsules, resulted in no significant increase of serum progesterone levels. In the hypophysectomized estrogen-treated rabbit, the rate-limiting step may be shifted from AC to another enzyme(s) which is (are) regulated by estrogen, allowing for partial (50%) pregnancy maintenance as reported by Spies *et al.* (47). Acute LH treatment of hypophysectomized rabbits with large doses of LH does not seem to sustain pregnancy (0% fetal survival, 47). Experiments testing whether subovulatory (non-desensitizing) doses of LH are able to maintain pregnancy in the hypophysectomized rabbit—a result expected from the above hypothesis—are currently in progress.

Prior to implantation, there is a lack of correlation between the responsiveness of the cyclase and serum progesterone levels (Fig. 9). In this first stage of CL-cyclase development (day 2 to day 6), LH-stimulated AC activity goes through a burst both in the pregnant and in the PSP animals (Figs.

[4] Hunzicker-Dunn and Birnbaumer, presented at the 8th Annual Meeting of the Society for the Study of Reproduction, Fort Collins, Colorado (July 22–25, 1975).

1,2,6), while progesterone levels increase slowly. Miller and Keyes (54) reported that ectopic CL on the kidney of ovariectomized rabbits (follicles were transplanted beneath the kidney capsule 6 to 8 h after mating and allowed to luteinize there) were secreting progesterone on days 3 to 6 after mating. Progesterone levels were declining in ovariectomized transplanted rabbits on day 10, but in those which had received an estrogen implant, progesterone levels were maintained. The results indicate that for the first 6 days following mating, progesterone synthesis by the rabbit CL is not dependent upon ovarian estrogens. It is possible that the pattern of responsiveness of the LH-stimulated AC system may also be autonomous, not only for the first 48 h (a time when luteal formation does not require pituitary support), but perhaps also until implantation (a time when luteal function is not dependent upon the trophic effects of estrogen). The AC may be programmed once an ovulatory level of LH reaches the Graafian follicle, and dependence of the AC on "external trophic factors" is acquired with the process of or the products from implantation. This is not to say that the CL does not require luteotrophic factors, since anti-LH after day 3 induces luteolysis (50).

If the first 6 days following coitus or hCG injection are, in fact, "preset," then the daily differences in the responsiveness of AC to LH (day 2 of PSP *vs* day 2 of pregnancy, for example) suggest that coitus and an hCG injection do not exert the same perturbations on the Graafian follicle. Ovulation occurs at the same time whether it is induced by exogenous or endogenous LH. One reported difference is that coitus induces not only LH release but also a release of PRL and ACTH (55). Although PRL has been reported to be luteotrophic along with LH (in tonic levels) and FSH and estrogen, the nature of its effect is not known (47). Secondly, serum progesterone levels in PSP never quite reach the same levels as during pregnancy, even before implantation (Fig. 9) (2,56), suggesting that additional influences, presumably of blastocyst or placental origin, may modulate the progesterone-synthetizing capacity of the estrogen-supported CL.

As discussed above, following implantation, there is a very close correlation between serum progesterone levels and the LH-stimulated AC activity of CL. Yet this correlation is never achieved in PSP, possibly because implantation never occurs. Peak progesterone levels are secreted during PSP between days 9 and 12, after which they steadily decline (56,51).

On day 9 of pregnancy, and on day 10 of PSP, there is a drastic but transient decline of AC activity for which we have no explanation. In the PSP rabbit CL (Fig. 6), LH-stimulated activity on day 9 differs from that on day 10 with a $P < 0.01$, and that on day 10 differs from the activity on day 11 with $P < 0.05$. In the absence of the data from PSP rabbits, one would speculate that the decline on day 9 in pregnant rabbits is due to natural decay of the CL, which is then stopped and reversed by some effect of implantation. Yet clearly, a similar effect seems to exist in PSP rabbits, indicating that some other factor, unrelated to implantation or ovum fertilization, must be responsible for the recovery of the AC system after days 9 and 10. Experiments on AC activity in corpora lutea induced on days 8, 9, or 10 may provide some insight into the reasons of this oscillatory behavior of the LH-sensitive system.

The findings that adenylyl cyclase activities change drastically during the preovulatory stage of the follicle and that LH-stimulated adenylyl cyclase may correlate with the functional activity of CL, suggest that the study of this enzyme system under various physiological and pharmacological conditions could provide new insights into the physiology of the ovary.

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